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DOSE-DEPENDENT CHANGES IN THE ANTIGENICITY OF BACTERIAL ENDOTOXIN EXPOSED TO IONIZING RADIATION

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SUMMARY The antigenic properties of the highly purified US reference standard endotoxin (RSE) exposed to varying doses of ionizing radiation were studied with double immunodiffusion, immunoelectrophoresis and immunoblotting. Rabbit RSE antisera identified 2 distinct major antigenic components for untreated RSE: one related to the O-polysaccharide side chain ("O-antigenic specificity"), the other to the R-core. Based on a serologic cross-reactivity of R-core of RSE (*Escherichia coli* 0113) with the R-core of the lipopolysaccharide from *E. coli* 0111, the core type of *E. coli* 0113 was identified as coli R3. Increasing exposure of RSE to ionizing radiation progressively destroyed all antigenic reactivities: at lower doses of radiation the rate of elimination differed for the 2 antigen classes. The O-polysaccharide was more sensitive to γ-radiation than the R-core and the O-antigenicity was lost before that of the R-core. Endotoxin molecules containing incomplete R-core (radiation-induced or mutant) did not react with the RSE antiserum.

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INTRODUCTION

FOR studying the relationship of structure to function for bacterial endotoxins (lipopolysaccharides; LPS), ionizing radiation has been shown to be a valuable tool. Previous work in our laboratory (1–4), and by others (5–8) demonstrated that ionizing radiation markedly alters the biological activities of LPS. We also showed this treatment causes dose-dependent changes in the chemical composition, molecular and "supramolecular" structure of endotoxin in an aqueous medium (1–4).

We report the changes in antigenic properties of a highly purified LPS, the US reference standard endotoxin (RSE), exposed to varying doses of ionizing radiation, using homologous rabbit anti-endotoxin sera. The changes in antigenic reactivity were compared to those occurring in the molecular structure of LPS as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

LPS Preparations

The source of the highly refined RSE was *Escherichia coli* (Braude strain) 0113:H10:K negative (9). RSE was prepared at a concentration of 2 mg/ml in sterile pyrogen-free water and dispensed into glass vials (2 ml each) by the Pharmaceutical Development Service of the Pharmacy Department, Clinical Center, NIH, Bethesda, MD. The vials were stored at −20°C until use. LPS from a smooth strain and J5 mutant of *E. coli* 0111:B4 and a smooth strain of *Salmonella typhimurium* were obtained from List Biological Laboratories, Campbell, CA. LPS from the PL2 mutant of *E. coli* K-12 (10), and *S. typhimurium* (SH 835) semirough (SR) LPS were kind gifts from Dr. W. Coleman of the NIH, Bethesda, MD, and Dr. P. H. Makela of the Central Public Health Laboratory, Helsinki, Finland, respectively. Lipooligosaccharide (LOS) was prepared by phenol-water extraction from *Neisseria meningitidis* M986 as described previously (11).

Exposure to Radiation

The glass vials containing RSE in water were exposed to ionizing radiation at a rate of 0·003 Mrad/min from a ^{60}Co-source. Radiation treatment was carried out at room temperature.

Anti-endotoxin Sera

Antibodies against untreated RSE were raised in 2 female New Zealand rabbits each weighing about 3 kg. The antigen (50 μg RSE) was mixed with complete Freund's adjuvant (Difco) in a final volume of 2 ml and this material was injected into the 2 rear foot pads, 2 sites subcutaneously and 2 sites intramuscularly in each animal on day 0. One, 2 and 8 weeks later, the rabbits received the same antigen dose with incomplete Freund's adjuvant (Difco) distributed into multiple sites as before. Blood was collected via the marginal ear vein prior to immunization, about 1 month (days 28, 29 and 30) and about 2½ months (days 79 and 80) after the onset of immunization. Approximately 5 ml of blood were drawn from each animal before immunization, whereas

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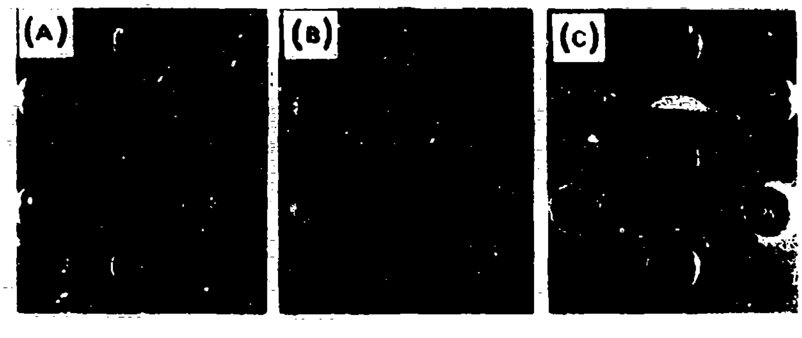

Figure 1. Double immunodiffusion patterns of untreated RSE, γ-irradiated RSE and heterologous LPS preparations with rabbit anti-RSE serum at 24 hr (a and c) and at 72 hr (b). Central wells (Ab) contain RSE antiserum (day 25, rabbit 29A). Peripheral wells contain 0·9% NaCl as a negative control (well 1) or LPS as antigen at a concentration of 2 mg/ml except when otherwise indicated. Well 2 = untreated RSE from *E. coli* 0113 (0 Mrad); wells 3–6 = RSE exposed to radiation doses of 0·18, 0·36, 1·08, and 2·88 Mrad, resp.; well 7 = smooth strain of *E. coli* 0111; well 8 = J5 mutant of *E. coli* 0111; well 9 = PL2 mutant of *E. coli* K-12; well 10 = smooth strain of *S. typhimurium*; and well 11 = semirough strain of *S. typhimurium* (0·2 mg/ml).

15–40 ml of blood were obtained on each subsequent occasion. The sera were heat-inactivated at 56°C for 30 minutes. When tested in double immunodiffusion (see below), pre-immune rabbit sera had no detectable RSE antibody activity. In contrast, all sera harvested at varying time periods following the onset of immunization exhibited some precipitating activity for the immunizing antigen, RSE. Rabbit antisera to *N. meningitidis* M986 LOS were raised by immunizing with purified LOS as described before (11).

Double Immunodiffusion

This technique was performed in 0·5% agarose (Sea Plaque agarose; Biomedical, Division of Marine Colloids, Inc., Rockland, ME) made up in Tris-HCl buffer, pH 8·2. LPS samples (usually 50 μg each in a volume of 25 μl) were placed into the peripheral wells and tested against rabbit anti-RSE serum (approximately 25 μl) placed into the central well.

Immunoelectrophoresis

Immunoelectrophoresis was carried out in commercial plates containing 1·5% agarose (Tital IV IE Plate; Helena Laboratories, Beaumont, Texas). LPS samples (40 μg each in a volume of 20 μl) were subjected to electrophoresis at 90 volt DC potential for 30 min in 0·05 M barbital buffer, pH 8·6 (Corning Special Barbital Buffer). The patterns were allowed to develop with rabbit anti-RSE sera for 24–72 hrs.

SDS-PAGE of Endotoxin

Electrophoretic analysis of LPS specimens was done in 14% polyacrylamide gel according to the method of Laemmli (12). To each site 0·25–5·0 μg of SDS-treated LPS was applied. After electrophoresis, a modified silver stain was used for the development of patterns (13).

Immunoblotting

The procedure of Towbin, Staehelin and Gordon (14) was modified for the electrophoretic blotting of LPS antigens. LPS were transferred from SDS-polyacrylamide gels onto nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH) by electrophoresis in 20 mM sodium phosphate buffer, pH 7·2 at 100 mA overnight in a Bio-Rad Trans-Blot cell. After treating the nitrocellulose membrane with 3% gelatin in Tris buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7·5) to block nonspecific reaction, the membrane was incubated with 100-fold diluted rabbit anti-endotoxin sera in TBS containing 1% gelatin for 3 hr. The LPS-antibody complexes were then reacted with goat anti-rabbit IgG-horseradish peroxidase conjugate in TBS containing 1% gelatin overnight. The antigen-antibody reactions were visualized by the purple insoluble product of the peroxidase from the substrates, 4-chloro-1-naphthol and hydrogen peroxide. The reagents used in the analysis were obtained from Bio-Rad Laboratories, Richmond, CA.

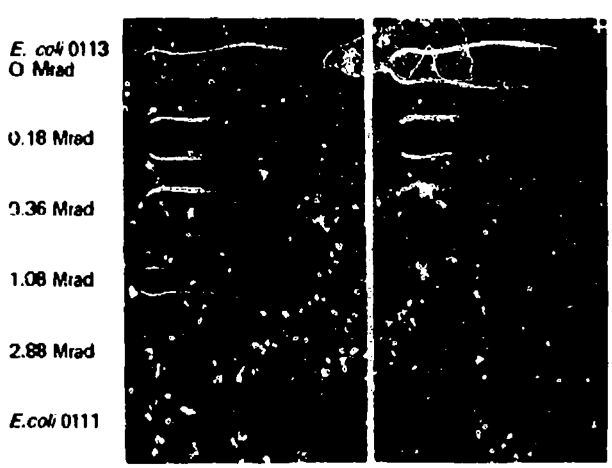

Figure 2. Immunoelectrophoretic patterns of untreated RSE, RSE exposed to ionizing radiation and heterologous endotoxin from a smooth strain of *E. coli* 0111 with rabbit RSE antiserum (day 29; rabbit 29A) at 24 hr (a) and 48 hr (b).

Results

Double Immunodiffusion in Agarose Plate

The rabbit RSE antiserum produced up to 3 precipitin lines with untreated and irradiated RSE in double immunodiffusion (Figures 1a and b). The appearance and density of the precipitin bands were dependent on the incubation time. By 24 hr only 2 precipitin lines were evident (Figure 1a). The denser line was located close to the antibody-containing well. The density of this line decreased with increasing doses of ionizing radiation to RSE, and the band disappeared at 2·88 Mrad. The second precipitin line (less dense) was more peripherally located (towards the antigen-containing wells) and was present even after exposure of RSE to 2·88 Mrad. Prolonged incubation of the diffusion plates for 48–72 hr enhanced the density of the second precipitin band of RSE irradiated with 0·36 and 1·08 Mrad (Figure 1b). In addition, a third weak precipitin line showing partial identity with the second line developed with endotoxin preparations exposed to 1·08 and 2·88 Mrad (Figure 1b).

Specificity studies with rabbit anti-RSE serum are shown in Figure 1c. The antiserum did not cross-react with smooth and semi-rough endotoxin preparations of *S. typhimurium*. Endotoxins purified from the O-

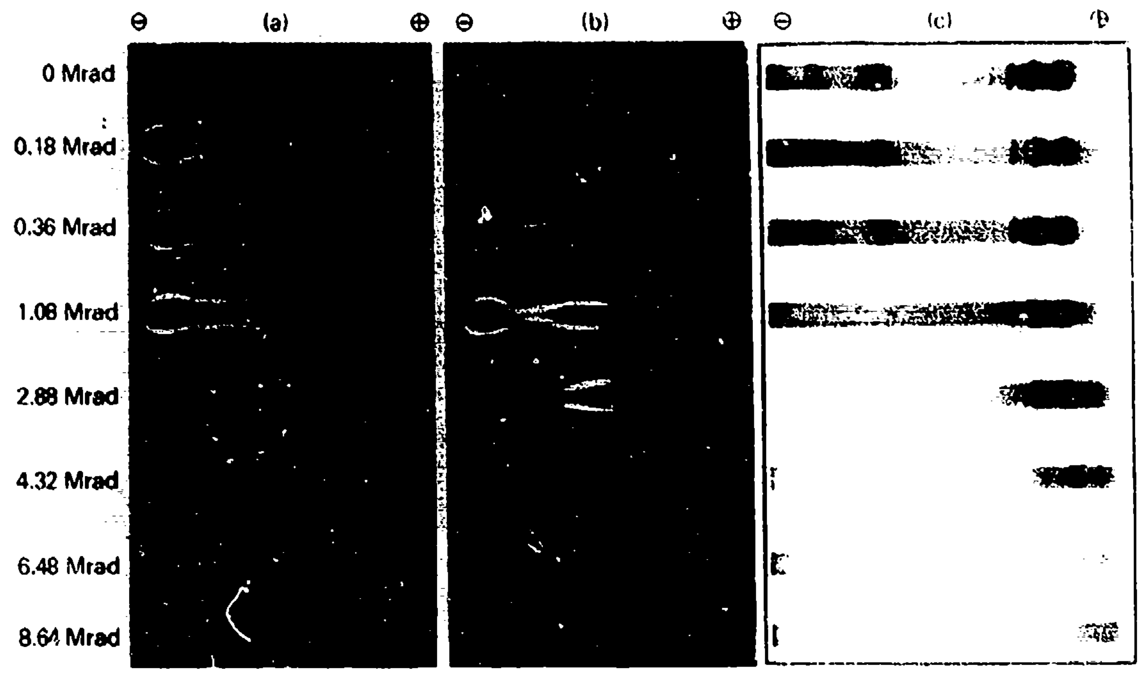

Figure 3. Immunoelectrophoretic patterns of untreated and γ-irradiated RSE specimens (40 µg each) with homologous rabbit antiserum (day 80; rabbit 29R) at 24 hr (a) and 48 hr (b); and silver stained SDS-PAGE patterns of the same RSE specimens (5 µg each) (c).

polysaccharide side chain deficient PL2 mutant of *E. coli* K-12 (13) and from the galactose epimerase deficient J5 mutant of *E. coli* 0111 (devoid not only of the repeating units of the O-polysaccharide chain but also a portion of the R-core) (15) also failed to produce any discernible precipitin band with rabbit antiserum for up to 72 hr. In contrast, a smooth LPS prepared from the 0111 serotype strain of *E. coli* showed cross-reactivity with the immunizing LPS (RSE) prepared from the 0113 strain of *E. coli*. However, the antigenic relationship with RSE appeared to involve only the outer precipitin line (Figure 1c).

Immunoeletrophoretic Studies

Due to the presence of phosphate, pyrophosphate and carboxyl groups, bacterial LPS are negatively charged macromolecules (16). Upon electrophoresis in agarose gel, the RSE molecules indeed behaved as anions and migrated towards the anode. When electrophoretically separated LPS components reacted with rabbit anti-RSE serum, 2 major precipitin bands developed with untreated RSE (Figures 2 and 3).

The longer precipitin band was located close to the antiserum containing trench extending from the application well to the anode, thus, including both slow and fast migrating antigen molecules. This band was sensitive to γ-radiation; its density rapidly diminished with progressively higher doses of radiation, and the band disappeared after exposure of RSE to 2·88 Mrad. Distortion and splitting of this band (suggesting heterogeneity) were evident in the immunoelectrophoretic pattern with both untreated and irradiated RSE specimens (Figures 2 and 3). Earlier antisera appeared to have a greater precipitating activity towards the antigen(s) present in this band (Figures 2 and 3).

The second major band became more distinct following radiation exposure of RSE specimens. With untreated RSE and RSE treated with low doses of radiation, this precipitin band ranged from the application well to midway to the anode, i.e., included relatively slow-migrating molecules (Figures 2 and 3). At higher doses of radiation (2·88 Mrad or greater), first the slower migrating component of this LPS fraction was eliminated, and all antigenic reactivity was destroyed with 6·48 Mrad (Figure 3).

Figure 2 shows that a cross-reactivity between RSE (prepared from the 0113 serotype strain of *E. coli*) and LPS prepared from the smooth strain of *E. coli* 0111 was present with rabbit anti-RSE sera in immunoelectrophoresis as well. The precipitin band of the *E. coli* 0111 LPS was comparable in position to the second precipitin line of the RSE.

Comparison of Immunoelectrophoresis and SDS–PAGE of Untreated and γ-irradiated RSE

We have shown previously (1–4) that, like other S-form LPS (17–18), untreated RSE shows molecular heterogeneity on SDS–PAGE. The fastest migrating band is composed of only complete R-core and lipid A (Figure

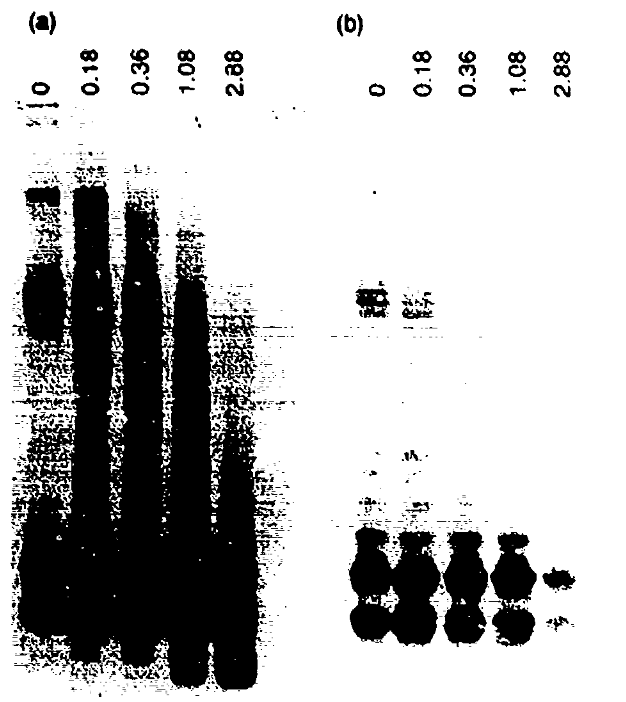

Figure 4. Silver stained SDS-PAGE (a) and antigenic reactivity (b) patterns of untreated and γ-irradiated RSE specimens. Each lane contains a nominal amount of 5 μg of RSE. For immunoblotting (b) RSE antiserum was from rabbit 29 R (day 29).

3c). The second band consists of a single unit of O-side chain polysaccharide, R-core and lipid A. The progressively slower migrating bands contain, in addition to R-core and lipid A, increasing numbers of repeating O-side chain units. Based on the density of staining, untreated RSE is distributed almost equally between the 2 fastest migrating bands (one is composed of R-core and lipid A, the other is composed of a single O-side chain unit, R-core and lipid A) and contains only a small amount of molecules with long O-polysaccharide side chain (Figure 3c).

The changes in antigenicity and molecular composition of radiation-treated RSE specimens correlated with each other (Figure 3). The disappearance of the slow-migrating endotoxin molecules with long O-polysaccharide chain on SDS-PAGE and the disappearance of the long precipitin line in immunoelectrophoresis occurred simultaneously. Likewise, the disappearance of the fasting migrating LPS molecules (composed of R-core and lipid A) on SDS-PAGE coincided with the disappearance of the second precipitin band in immunoelectrophoresis.

Immunoblotting Studies

Rabbit RSE antiserum reacted only with RSE molecules containing O-polysaccharide side chain and/or complete R-core in the sensitive immunoblotting technique (Figures 4a and b). Radiation treatment of RSE primarily eliminated the O-polysaccharide side chain related antigenic reactivity. The fastest migrating component (consisting of incomplete R-core and lipid A) of irradiated RSE specimens showed no reactivity with the RSE antiserum (Figures 4a and b). For comparison, the reaction between rabbit anti-meningococcal M986 LOS serum and meningococcal M986 endotoxin (LOS) that is deficient in O-polysaccharide side chain (13) was studied. Like irradiated RSE, the fastest migrating molecules of LOS (composed of incomplete R-core and lipid A) (Figure 5c) were nonreactive to the homologous antiserum (Figure 5d).

When the RSE antiserum was tested against heterologous unirradiated LPS preparations, only LPS

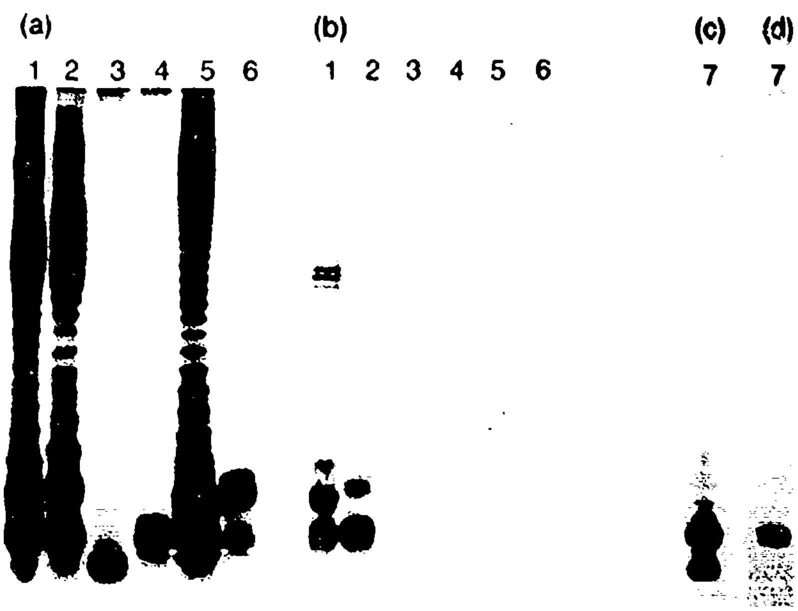

Figure 5. Silver stained SDS-PAGE (a and c) and antigenic reactivity (b and d) patterns of untreated LPS preparations. RSE (from *E. coli* O113) (lane 1), *E. coli* O111 (lane 2) and *S. typhimurium* (lane 5) smooth LPS preparations were applied at 5 μg/lane for both silver staining and immunoblotting. LPS from *E. coli* J⁻ (lane 3), PL2 (lane 4) and *S. typhimurium* semi- (lane 6) mutants, and from *N. meningitidis* N... (lane 7) were applied at 0·25 μg/lane for silver staining and at 0·5 μg/lane for immunoblotting. For immunoblotting, rabbit RSE antiserum (29 R; day 29) and rabbit M986 meningococcal antiserum were used for (b) and (d), resp.

prepared from the smooth strain of *E. coli* 0111 showed cross-reactivity (Figure 5b). This cross-reactivity apparently involved R-core rich LPS molecules which were small and, consequently, fast migrating molecular species (containing no or only 1 O-polysaccharide side chain unit) in SDS–PAGE (Figure 5a). LPS from the partially R-core deficient J5 mutant of *E. coli* 0111, the O-side chain deficient PL2 mutant of *E. coli* K-12, and smooth and semi-rough (long O-side chain deficient) strains of *S. typhimurium* failed to produce any cross-reaction with the RSE antiserum (Figures 5a and b).

Discussion

Previous reports document 2 to 3 LPS components that are precipitable in agar gel with antisera raised with whole bacteria (19, 20). It was also observed that "haptene" (i.e., polysaccharide moiety) from LPS precipitates only about 50% of the antibody from rabbit antityphoid (LPS) serum (19, 20). In contrast, absorption of the antiserum with purified LPS resulted in the loss of both precipitation lines in immunodiffusion (19, 20). Our findings are in accord with these observations. Using antisera raised with a highly refined LPS preparation (RSE), we found 2 distinct major antigenic components with double immunodiffusion in the untreated immunizing antigen. In addition, a third minor antigenic component exhibiting partial identity with one of the major components was noted after exposure of RSE to high doses of ionizing radiation. We identified the immunoreactive LPS components and the respective antibodies in 2 ways: (a) by comparing the reaction patterns of untreated and γ-irradiated RSE specimens with anti-RSE sera using double immuno-diffusion, immunoelectrophoresis, and immunoblotting techniques to silver staining in SDS–PAGE, and (b) by evaluating the antigenic cross-reactivities between heterologous LPS and anti-RSE sera.

Comparisons among the 4 techniques are consistent for the proposed structure of the RSE molecule and fragments. The inner (close to the antibody well) precipitin line in double immunodiffusion and the long band in immunoelectrophoresis apparently represent LPS molecules containing O-polysaccharide (O-antigen) and the respective (O-antigen specific) antibodies. In turn, the outer (close to the antigen well) precipitin band in double immunodiffusion and the slow-migrating relatively short band in immunoelectrophoresis most likely consist of R-core "rich" LPS molecules (that contain no O-polysaccharide chain or only one repeating unit) and the respective (R-core specific) antibodies. The antigenic cross-reactivity of *E. coli* 0111:B4 S-form LPS with RSE (*E. coli* 0113:H10:K negative) can be explained by the presence of cross-reactive R-core antibodies in rabbit anti-RSE sera. It was noted that the R-core may be structurally and antigenically similar among several strains within a genus (21).

The core type of LPS from the *E. coli* 0113 strain (RSE) has not yet been reported (for review see ref. 22). Our finding of R-core related antigenic cross-reactivity with RSE antiserum between RSE (*E. coli* 0113) and LPS from *E. coli* 0111 with a core type of coli R3 (23) indicates that the core type of *E. coli* 0113 also is coli R3. The absence of antigenic cross-reactivity between RSE and LPS from the PL-2 mutant of *E. coli* K-12 with a core type of coli K-12 (24) or LPS from smooth and SR strains of *S. typhimurium* with a core type of Ra (25) is consistent with their different core types.

The lack of reactivity between RSE (*E. coli* 0113) antisera and LPS containing incomplete R-core such as irradiated homologous LPS or untreated heterologous LPS from the Rc-like J5 mutant of *E. coli* 0111:B4 (deficient in synthesis of complete R-core but expressing complete lipid A) indicates that (a) antibodies directed against complete R-core in RSE antiserum do not recognize molecules containing incomplete R-core, and (b) little or no precipitating lipid A antibodies were produced upon immunization with purified RSE.

A high specificity of core antibodies to the core structure of the immunizing LPS was seen in other experiments as well. Like RSE antisera, antimeningococcal LOS sera did not react with homologous LOS molecules containing incomplete R-core (11). Rabbit antibodies prepared by immunizing with different *Salmonella* R form LPS (Rb_2, Rc, and Re) exhibited distinct serological specifities, indicating that the R-oligosaccharides can be classified serologically, as can the respective mutants (chemotypes) (26). In turn, rabbit and human polyclonal antibodies to the Rc-like J5 LPS core and R form LPS of *Salmonellae* have been claimed to show extensive cross-reactivity with and *in vivo* protection against heterologous LPS preparations and the respective bacteria (27–31). Recent work with murine monoclonal antibodies to *E. coli* J5 endotoxin, however, demonstrated greater cross-reactivity when assayed against the whole bacterium than when assayed against the corresponding purified endotoxin (32). In addition, no protection was seen against lethal LPS challenge when the monoclonal antibodies were given to mice (32).

The lack of detectable amounts of precipitating lipid A antibodies in anti-RSE sera is likely related to the poor immunogenicity and cryptic position of lipid A in LPS aggregates. Smooth and R-form LPS neither react with nor induce lipid A antibodies (21, 27, 28).

Our previous (1–4) and present results show that ionizing radiation preferentially destroys the O-polysaccharide chain. Consequently, the O-antigenicity is eliminated at a faster rate than the antigenicity related to the R-core. The finding of a differential effect of ionizing radiation on S-form LPS antigenic determinants is in good agreement with the results of Previte, Chang and

El-Bisi (5). In a mouse protection test these authors observed that ionizing radiation more effectively destroys the specific determinants of LPS that are responsible for "specific antibody" production (measured by protection after challenge 6 days post-vaccination) than those which elicit "nonspecific resistance" production (measured by protection after challenge 1 day post-vaccination).

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